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(54) Title: ADENOVIRAL TARGETING AND MANIPULATION OF IMMUNE SYSTEM RESPONSE USING TARGETING PEPTIDES

(57) Abstract: The present invention concerns compositions and methods relating to the identification and use of targeting peptides. Such targeting peptides selectively home to specific organs or tissues *in vivo*. The novel targeting sequences disclosed herein are of use for the targeted delivery of various therapeutic agents to the targeted organ or tissue. In particular embodiments, the present invention concerns bispecific targeting reagents comprising an organ targeting peptide attached to a molecule, such as a Fab fragment, that binds to a gene therapy vector or other therapeutic agent. In alternative embodiments, bispecific targeting peptides containing an organ targeting moiety and a gene therapy or therpeutic agent targeting moiety may be obtained and used for targeted delivery. Other embodiments concern modulation of host immune system function through the targeted delivery of antigens or other molecules to lymph nodes. Numerous examples of targeting peptide sequences against adenovirus or lymph node tissue are disclosed herein.

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ADENOVIRAL TARGETING AND MANIPULATION OF IMMUNE SYSTEM RESPONSE USING TARGETING PEPTIDES

BACKGROUND OF THE INVENTION

This application claims priority from U.S. Provisional Patent Application No. 60/231,266 filed September 8, 2000, and U.S. Patent Application No. 09/765,101, filed January 17, 2001. This invention was made with government support under grants DAMD 17-98-1-8041 and 17-98-1-8581 from the U.S. Army and grants 1R01CA78512-01A1, 1R01CA90810-01 and 1R01CA82976-01 from the National Institutes of Health. The government has certain rights in this invention.

1. Field of the Invention

The present invention concerns the fields of molecular medicine and targeted delivery of therapeutic agents. More specifically, the present invention relates to compositions and methods for targeting of therapeutic vectors, particularly adenoviral vectors, using bispecific targeting reagents and to modulation of immune system response using lymph node targeting peptides.

2. Description of Related Art

Therapeutic treatment of many human disease states is limited by the systemic toxicity of the therapeutic agents used. Cancer therapeutic agents in particular exhibit a very low therapeutic index, with rapidly growing normal tissues such as skin and bone marrow affected at concentrations of agent that are not much higher than the concentrations used to kill tumor cells. Treatment of cancer and other organ or tissue confined disease states would be greatly facilitated by the development of compositions and methods for targeted delivery to a desired organ or tissue of a therapeutic agent.

Recently, an *in vivo* selection system was developed using phage display libraries to identify organ or tissue targeting peptides in a mouse model system. Phage display libraries expressing transgenic peptides on the surface of bacteriophage were initially developed to map epitope binding sites of immunoglobulins (Smith, 1985; Smith and Scott, 1985, 1993). Such libraries can be generated by inserting random

oligonucleotides into cDNAs encoding a phage surface protein, generating collections of phage particles displaying unique peptides in as many as 10^9 permutations. (Pasqualini and Ruoslahti, 1996, Arap et al, 1998a; Arap et al 1998b).

Intravenous administration of phage display libraries to mice was followed by the recovery of phage from individual organs (Pasqualini and Ruoslahti, 1996). Phage were recovered that were capable of selective homing to the vascular beds of different mouse organs or tissues, based on the specific targeting peptide sequences expressed on the outer surface of the phage (Pasqualini and Ruoslahti, 1996). A variety of organ and tumor-homing peptides have been identified by this method (Rajotte et al., 1998, 1999; Koivunen et al., 1999; Burg et al., 1999; Pasqualini, 1999). Each of those targeting peptides bound to different receptors that were selectively expressed on the vasculature of the mouse target tissue (Pasqualini, 1999; Pasqualini et al., 2000b; Folkman, 1995; Folkman 1997). Tumor-homing peptides bound to receptors that were upregulated in the tumor angiogenic vasculature of mice (Brooks et al., 1994; Pasqualini et al., 2000b). In addition to identifying individual targeting peptides selective for an organ or tissue (Pasqualini and Ruoslahti, 1996; Arap et al, 1998a; Koivunen et al., 1999), this system has been used to identify endothelial cell surface markers that are expressed in mice *in vivo* (Rajotte and Ruoslahti, 1999).

Attachment of therapeutic agents to targeting peptides resulted in the selective delivery of the agent to a desired organ or tissue in the mouse model system. Targeted delivery of chemotherapeutic agents and proapoptotic peptides to receptors located in tumor angiogenic vasculature resulted in a marked increase in therapeutic efficacy and a decrease in systemic toxicity in tumor-bearing mouse models (Arap et al., 1998a, 1998b; Ellerby et al., 1999).

Attempts have been made to target delivery of gene therapy vectors to specific organs or tissues. Directing such vectors to the site of interest would enhance therapeutic effects and diminish adverse systemic immunologic responses. Adenovirus type 5 (Ad5)-based vectors have been commonly used for gene transfer studies (Weitzman *et al.*, 1997; Zhang, 1999). The attachment of Ad5 to the target cell is

mediated by the capsid's fiber knob region, which interacts with cell surface receptors, including the coxsackie adenovirus receptor (CAR) and possibly with MHC class I (Bergelson *et al.*, 1997; Hong *et al.*, 1997). Upon systemic administration *in vivo*, binding of virus to CAR can result in unintended enrichment of vectors in non-targeted but CAR-expressing tissues. Conversely, target cells that express little or no CAR are inefficiently transduced. A need exists to retarget Ad5 vectors to allow more selective delivery of gene therapy.

Previous efforts to accomplish adenoviral retargeting have used bispecific conjugates or genetically modified capsids. Bispecific conjugates were used to target Ad5 to growth factor receptors that are up-regulated during tumor development or inflammation (Douglas *et al.*, 1996; Goldman *et al.*, 1997; Watkins *et al.*, 1997; Miller *et al.*, 1998). Ad5 re-targeting was also attempted using ligands for CD3, αv integrins or heparan sulfate receptors (Wickham *et al.*, 1995; 1996*a*, 1996*b*; 1997*a*, 1997*b*; Vigne *et al.*, 1999). In other attempts, heterologous ligands were incorporated into the HI loop of the fiber knob to attempt to retarget adenovirus (Dmitriev *et al.*, 1998; Krasnykh *et al.*, 1998). Heterologous ligands also have been incorporated into the envelopes of retroviruses or the capsids of adenoviruses and adeno-associated viruses, thereby targeting these vectors to integrins (Dmitriev et al., 1998; Vigne et al., 1999; Girod et al., 1999), T-cell receptors (Engelstadter et al., 2000) or melanoma-associated antigens (Martin et al., 1999). These approaches suffered from two major limitations: (1) the targeted receptors were not restricted to specific tissues; or (ii) the targeted receptors were not selectively expressed on the lumenal surface of endothelial cells lining blood vessels, a requirement for intravenously administered therapies. A need exists in the art for an effective means of retargeting adenoviral and other gene therapy vectors to allow selective intravenous delivery to organs or tissues of interest.

The targeted delivery of immunogenic agents to portions of the host immune system, such as spleen and lymph node, to modulate immune system response has been attempted. Such approaches have included direct inoculation of antigens into lymph nodes or spleen (Sigel *et al.*, 1983; Nilsson *et al.*, 1987) and targeting of receptors located on dentritic and other antigen-presenting cells (Wang *et al.*, 2000; Heijnen *et*

al., 1996). Results to date have been only marginally successful. A need exists for an efficient and effective method of immune system targeting of immunogenic agents.

SUMMARY OF THE INVENTION

The present invention solves a long-standing need in the art by providing compositions and methods for the selective delivery of gene therapy vectors, including but not limited to adenoviral vectors, to specific organs or tissues *in vivo*. In other embodiments, the compositions and methods allow the targeting of lymph node tissues for delivery of various antigens, thereby modulating the host's immune system response to the antigen. The skilled artisan will realize that the scope of the claimed methods of use is not limited as to the type of antigen that could be targeted, but rather includes any immunogenic compound ranging from a single purified molecule to a pathogenic agent such as a virus, bacterium, or a diseased host cell. In additional embodiments, the lymph node targeted compound may include one or more regulatory molecules for immune system function, including but not limited to cytokines or chemokines. In certain embodiments, the diseased cells or pathogenic organisms to be treated may be located at a distant site in the body from the targeted organ or tissue. A non-limiting example of such a method of use would be to treat metastatic cancer by provoking a systemic humoral immune response to a tumor-associated antigen, amplifying the host immune response to the tumor.

Certain embodiments of the invention concern methods of targeted delivery comprising selecting a targeting peptide for a desired organ or tissue, attaching said targeting peptide to a molecule, macromolecular complex or gene therapy vector, and providing said peptide attached to said molecule, complex or vector to a subject. In certain preferred embodiments, the organ or tissue is lymph node. Preferred examples of lymph node targeting peptides are disclosed in Example 2 below.

Another embodiment of the present invention concerns molecular adaptors for targeted gene therapy. In a preferred embodiment, the molecular adaptor comprises a Fab fragment of an antibody that is specific for a gene therapy vector, covalently attached to a targeting peptide sequence that provides selective targeting to a desired

organ or tissue. In a more preferred embodiment, the gene therapy vector is an adenovirus, particularly a type 5 adenovirus. The skilled artisan will realize that the present invention is not limited to adenovirus vectors, but may include any gene therapy vector that is known in the art. Similarly, the vector binding portion of the molecular adaptor is not limited to Fab fragments of antibodies, but may include other molecules that can be used to attach a targeting peptide to a gene therapy vector. The only requirement is that the gene therapy vector should be selectively targeted to a desired organ or tissue in the presence of the molecular adaptor. Preferred examples of such Fab fragments and adenoviral targeting peptides are provided in Example 1 below.

A further embodiment concerns compositions and methods for modulation of the humoral immune response by targeting antigens to lymph nodes. Lymph node targeting peptides may be attached to antigens and administered to a subject. The presence of the targeting peptides provides for selective delivery of the antigen to the lymph node, where it modulates the humoral immune response of the subject. In preferred embodiments, the targeting peptide is covalently attached to the antigen. Antigens within the scope of the invention may include any molecule or macromolecular assemblage that is capable of provoking a humoral immune response in a subject, including but not limited to peptides, proteins, glycoproteins, lipoproteins, viruses, bacteria, pathogenic microorganisms and diseased cells.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Monoclonal antibodies neutralized Ad5 infection of HeLa cells. Ad5-infection neutralization assays were performed on HeLa cells. The number of positive cells observed in wells infected with Ad5-LacZ alone (no antibody) was set as 100%. Data are means of four different data sets in two independent experiments. Standard deviations were <10% of the mean.

FIG. 2. A phage displaying the GFE-1 peptide bound to RD and MDA-MB-435 cells. RD and MDA-MB-435 cells were grown and incubated with equal amounts of GFE-1 phage or the insertless fd-tet phage. Phage were recovered by bacterial infection and the number of transducing units (TU) was determined by colony counting.

FIG. 3. Characterization of the Fab-GFE adaptor conjugate. The Fab-GFE adaptor neutralized Ad5 infection of HeLa cells. Ad5-infection neutralization assays were performed on HeLa cells. The number of positive cells observed in wells infected with Ad5-LacZ alone (no antibody) was set as 100%. Data points are means of two independent experiments.

FIG. 4. The Fab-GFE adaptor conjugate inhibited membrane dipeptidase function. RD cells were grown and lysed. Cell lysate equivalent to 10 μg protein was incubated with the Fab-GFE adaptor for 15 min followed by addition of the MDP substrate Gly-D-Phe. Enzyme activity was detected fluorometrically at various time points after addition of the developer. Data points represent means of triplicates of one representative experiment.

FIG. 5. Targeting of Ad5 vectors to RD cells and MDA-MB-435 cells with a 1C5IIE11-based Fab-GFE adaptor. RD and MDA-MB-435 cells were infected with Ad5-LacZ or Ad5-GFP at an MOI of 150 PFU/cell in the presence or absence of 1C5IIE11-based Fab alone, Fab-GFE, or Fab-CARAC conjugates. Adaptor or Fab concentrations were 12.5 μg/ml. To estimate an EC_{50} for the Fab-GFE adaptor on RD or MDA-MB-435 cells, cells were incubated with Ad5-LacZ in presence or absence of Fab-based adaptors in a range of concentrations. Cells were stained after 24 h for ß-galactosidase expression and positive cells were counted upon microscopic observation. Data points represent means of duplicates from one of four independent experiments revealing similar results.

FIG. 6. Efficiency of binding to Ad5 of phage displaying adenovirus targeting peptides.

FIG. 7. Phage displaying PTCAYGWCA (SEQ ID NO:7) or WSCARPLCG (SEQ ID NO:8) peptides elicited a stronger immune response than an untargeted (insertless) fd-tet control phage. Phage were injected intravenously into female 2-month old Balb/C mice (2 mice were used per amount of phage to be injected) and the anti-phage antibody titer was determined by serum collection and ELISA on immobilized fd-tet phage 3 days after vaccination. Data represent optical density values (ODs) of the p-nitrophenyl phosphate substrate at 405 nm in ELISAs after two immunizations. The serum dilution was 1:500.

FIG. 8. Enhancement of the humoral immune response by lymph node targeting is specific and can be blocked by the cognate synthetic peptides. Mice were vaccinated with PTCAYGWCA (SEQ ID NO:7) phage, WSCARPLCG (SEQ ID NO:8) phage, or insertless fd-tet control phage. An additional set of mice vaccinated with either PTCAYGWCA (SEQ ID NO:7) phage or WSCARPLCG (SEQ ID NO:8) phage was injected with 1 mg of the cognate peptide (synthesized by Anaspec, Inc., CA) 5 min prior to vaccination. Anti-phage antibody titer was determined. OD values represent means of triplicates + SEM in ELISAs performed with sera from five mice after three vaccinations. Serum dilution was 1:500; pre-immune normal mouse serum (NMS) served as control. * $p = 0.004$; ** $p = 0.001$ (Student *t*-test)

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more of an item.

A "targeting peptide" is a peptide comprising a contiguous sequence of amino acids that is characterized by selective localization to an organ or tissue. Selective localization may be determined, for example, by methods disclosed below, wherein the putative targeting peptide sequence is incorporated into a protein that is displayed on the outer surface of a phage. Administration to a subject of a library of such phage that have been genetically engineered to express a multitude of such targeting peptides of

different amino acid sequence is followed by collection of one or more organs or tissues from the subject and identification of phage found in that organ or tissue. A phage expressing a targeting peptide sequence is considered to be selectively locallized to a tissue or organ if it exhibits greater binding in that tissue or organ compared to a control tissue or organ. Alternatively, a phage expressing a targeting peptide sequence that exhibits selective localization should show an increased enrichment in the target organ compared to a control organ when phage recovered from the target organ are reinjected into a second host for another round of screening. Further enrichment may be exhibited following a third round of screening. Another alternative means to determine selective localization is that phage expressing the putative target peptide exhibit at least a two-fold, more preferably at least a three-fold enrichment in the target organ compared to control phage that express a non-specific peptide or that have not been genetically engineered to express any putative target peptides. Another means to determine selective localization is that locallization to the target organ of phage expressing the target peptide is at least partially blocked by the co-administration of a synthetic peptide containing the target peptide sequence. "Targeting peptide" and "homing peptide" are used synonymously herein.

A "phage display library" means a collection of phage that have been genetically engineered to express a set of putative targeting peptides on their outer surface. In preferred embodiments, DNA sequences encoding the putative targeting peptides are inserted in frame into a gene encoding a phage capsule protein. In other preferred embodiments, the putative targeting peptide sequences are in part random mixtures of all twenty amino acids and in part non-random. In certain preferred embodiments the putative targeting peptides of the phage display library exhibit one or more cysteine residues at fixed locations within the targeting peptide sequence.

A "macromolecular complex" refers to a collection of molecules that may be random, ordered or partially ordered in their arrangement. The term encompasses biological organisms such as bacteriophage, viruses, bacteria, unicellular pathogenic organisms, multicellular pathogenic organisms and prokaryotic or eukaryotic cells. The term also encompasses non-living assemblages of molecules, such as liposomes,

microcapsules, microparticles and magnetic beads. The only requirement is that the complex contains more than one molecule. The molecules may be identical, or may differ from each other.

A "receptor" for a targeting peptide includes but is not limited to any molecule or complex of molecules that binds to a targeting peptide. Non-limiting examples of receptors include peptides, proteins, glycoproteins, lipoproteins, epitopes, lipids, carbohydrates, multi-molecular structures, a specific conformation of one or more molecules and a morphoanatomic entity. In preferred embodiments, a "receptor" is a naturally occurring molecule or complex of molecules that is present on the lumenal surface of endothelial cells lining blood vessels within a target organ or tissue.

Phage Display

The methods described herein for identification of targeting peptides involve the *in vivo* administration of phage display libraries. Various methods of phage display and methods for producing diverse populations of peptides are well known in the art. For example, U.S. Pat. Nos. 5,223,409; 5,622,699 and 6,068,829, each of which is incorporated herein by reference, disclose methods for preparing a phage library. The phage display technique involves genetically manipulating bacteriophage so that small peptides can be expressed on their surface (Smith, 1985; Smith and Scott, 1985, 1993). The potential range of applications for this technique is quite broad, and the past decade has seen considerable progress in the construction of phage-displayed peptide libraries and in the development of screening methods in which the libraries are used to isolate peptide ligands. For example, the use of peptide libraries has made it possible to characterize interacting sites and receptor-ligand binding motifs within many proteins, such as antibodies involved in inflammatory reactions or integrins that mediate cellular adherence. This method has also been used to identify novel peptide ligands that serve as leads to the development of peptidomimetic drugs or imaging agents (Arap *et al.*, 1998a). In addition to peptides, larger protein domains such as single-chain antibodies can also be displayed on the surface of phage particles (Arap *et al.*, 1998a).

The most efficient amino acid sequences for targeting a given organ or tissue can be isolated by "biopanning" (Pasqualini and Ruoslahti, 1996; Pasqualini, 1999). In brief, a library of phage containing putative targeting peptides is administered to a subject and samples of organs or tissues containing phage are collected. In preferred embodiments utilizing filamentous phage, the phage may be propagated *in vitro* between rounds of biopanning in pilus-positive bacteria. The bacteria are not lysed by the phage but rather secrete multiple of copies of phage that display a particular insert. Phage that bind to a target molecule can be eluted from the target organ or tissue and then amplified by growing them in host bacteria. If desired, the amplified phage can be administered to a host and samples of organs or tissues again collected. Multiple rounds of biopanning can be performed until a population of selective binders is obtained. The amino acid sequence of the peptides is determined by sequencing the DNA corresponding to the targeting peptide insert in the phage genome. The identified targeting peptide can then be produced as a synthetic peptide by standard protein chemistry techniques (Arap *et al.*, 1998a, Smith and Scott, 1985). This approach allows circulating targeting peptides to be detected in an unbiased functional assay, without any preconceived notions about the nature of their target. Once a candidate target is identified as the receptor of a targeting peptide, it can be isolated, purified and cloned by using standard biochemical methods (Pasqualini, 1999; Rajotte and Ruoslahti, 1999).

Choice of phage display system.

Previous *in vivo* selection studies performed in mice preferentially employed libraries of random peptides expressed as fusion proteins with the gene III capsule protein in the fUSE5 vector (Pasqualini and Ruoslahti, 1996). The number and diversity of individual clones present in a given library is a significant factor for the success of *in vivo* selection. It is preferred to use primary libraries, which are less likely to have an over-representation of defective phage clones (Koivunen *et al.*, 1999). The preparation of a library should be optimized to between 10^8-10^9 transducing units (T.U.)/ml. In certain embodiments, a bulk amplification strategy is applied between each round of selection.

Phage libraries displaying linear, cyclic, or double cyclic peptides may be used within the scope of the present invention. However, phage libraries displaying 3 to 10 random residues in a cyclic insert ($CX_{3-10}C$) are preferred, since single cyclic peptides tend to have a higher affinity for the target organ than linear peptides. Libraries displaying double-cyclic peptides (such as $CX_3C\ X_3C\ X_3C$; Rojotte *et al.*, 1998) have been successfully used. However, the production of the cognate synthetic peptides, although possible, can be complex due to the multiple conformers with different dissulfide bridge arrangements .

Identification of homing peptides and receptors by in vivo phage display in mice.

In vivo selection of peptides from phage-display peptide libraries administered to mice has been used to identify targeting peptides selective for normal mouse brain, kidney, lung, skin, pancreas, retina, intestine, uterus, prostate, and adrenal gland (Pasqualini and Ruoslahti, 1996; Pasqualini, 1999; Rajotte *et al.*, 1998). These results show that the vascular endothelium of normal organs is sufficiently heterogenous to allow differential targeting with peptide probes (Pasqualini and Ruoslahti, 1996; Rajotte *et al.*, 1998). A means of identifying peptides that home to the angiogenic vasculature of tumors has been devised, as described below. A panel of peptide motifs that target the blood vessels of tumor xenografts in nude mice has been assembled (Arap *et al.*, 1998a; reviewed in Pasqualini, 1999). These motifs include the sequences CDCRGDCFC (SEQ ID NO:25) (termed RGD-4C), NGR, and GSL. The RGD-4C peptide has previously been identified as selectively binding αv integrins and has been shown to home to the vasculature of tumor xenografts in nude mice (Arap *et al.*, 1998a, 1998b; Pasqualini *et al.*, 1997).

The receptors for the tumor homing RGD4C targeting peptide has been identified as αv integrins (Pasqualini *et al.*, 1997). The αv integrins play an important role in angiogenesis. The $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are absent or expressed at low levels in normal endothelial cells but are induced in angiogenic vasculature of tumors (Brooks *et al.*, 1994a, 1994b; Hammes *et al.*, 1996). Aminopeptidase N/CD13 has

recently been identified as an angiogenic receptor for the NGR motif (Burg *et al.*, 1999). Aminopeptidase N/CD13 is strongly expressed not only in the angiogenic blood vessels of prostate cancer in mice but also in the normal epithelial prostate tissue. Table 1 shows representative ligand-receptor pairs for tumor targeting by *in vivo* phage display in mice.

Table 1. Cell surface receptors and homing motifs isolated by *in vivo* phage display

Receptor		*Carrier ?*	*Localization*	*Homing motif*
αv integrins	Cell Adhesion	Yes	EC, tumor cells	RGD4C
CD13	Protease	Yes	EC, pericytes, tumor	CNGRC
Aminopeptidase A	Protease	N/D	Pericytes	CPRECES
NG2/HMWMAA	Proteo-Glycan	N/D	Pericytes, tumor cells	GSL
MMP-2/MMP-9	Protease	Yes	EC, tumor cells	CTTHWGFTLC
MDP	Protease	N/D	EC	CGFEC

HMWMAA, high molecular weight melanoma-associated antigen; MMP, matrix metalloproteinase; EC, endothelial cells; N/D, not determined; R, arginine; G, glycine; C, cysteine; N, asparagine; P, proline; S, serine; L, leucine; T, threonine, H, histidine; W, tryptophan, F, phenylalanine; MDP, membrane dipeptidase.

Tumor-homing phage co-localize with their receptors in the angiogenic vasculature of tumors but not in non-angiogenic blood vessels in normal tissues (Arap *et al.*, 1998b). Immunohistochemical evidence shows that vascular targeting phage bind to tumor blood vessels in tissue sections (Pasqualini *et al.*, 2000b) but not to normal blood vessels. A negative control phage with no insert (fd phage) did not bind to normal or tumor tissue sections. The expression of the angiogenic receptors was evaluated in cell lines, in non-proliferating blood vessels and in activated blood vessels of tumors and other angiogenic tissues such as corpus luteum. The angiogenic receptors were not detected in the vasculature of normal organs.

The distribution of these receptors was analyzed by immunohistochemistry in tumor cells, tumor vasculature, and normal vasculature. Alpha v integrins, CD13, aminopeptidase A, NG2, and MMP-2/MMP-9 - the known receptors in tumor blood vessels - are specifically expressed in angiogenic endothelial cells and pericytes. Angiogenic neovasculature expresses markers that are either expressed at very low levels or not at all in non-proliferating endothelial cells (not shown).

The markers of angiogenic endothelium include receptors for vascular growth factors, such as specific subtypes of VEGF and basic FGF receptors, and αv integrins, among many others (Mustonen and Alitalo, 1995). Thus far, identification and isolation of novel molecules characteristic of angiogenic vasculature has been slow, mainly because endothelial cells undergo dramatic phenotypic changes when grown in culture (Watson *et al.*, 1995).

Many of these tumor vascular markers are proteases and some of the markers also serve as viral receptors. Alpha v integrins are receptors for adenoviruses (Wickham *et al.*, 1997c) and CD13 is a receptor for coronaviruses (Look *et al.*, 1989). MMP-2 and MMP-9 are receptors for echoviruses (Koivunen *et al.*, 1999). Aminopeptidase A also appears to be a viral receptor. Bacteriophage may use the same cellular receptors as eukaryotic viruses. These findings suggest that receptors isolated by *in vivo* phage display will have cell internalization capability, a key feature for utilizing the identified peptide motifs as targeted gene therapy carriers.

Targeted delivery

Peptides that home to tumor vasculature have been coupled to cytotoxic drugs or proapoptotic peptides to yield compounds that were more effective and less toxic than the parental compounds in experimental models of mice bearing tumor xenografts (Arap *et al.*, 1998a; Ellerby *et al*, 1999). As described below, the insertion of the RGD-4C peptide into a surface protein of an adenovirus has produced an adenoviral vector that may be used for tumor targeted gene therapy (Arap *et al.*, 1998b). In other embodiments, fusion proteins or chimeric proteins containing targeting peptides linked to various therapeutic proteins or peptides may be produced and administered by the

methods of the present invention. Non-limiting examples of therapeutic proteins or peptides that may be targeted for delivery by the disclosed methods are listed below.

Regulators of Programmed Cell Death

Apoptosis, or programmed cell death, is an essential process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr et al., 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse apoptotic stimuli (Cleary and Sklar, 1985; Cleary et al., 1986; Tsujimoto et al., 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2 protein now is recognized to be a member of a family of related proteins, which can be categorized as death agonists or death antagonists.

Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins that share structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (e.g., Bcl_{XL}, Bcl_W, Bcl_S, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (e.g., Bax, Bak, Bik, Bim, Bid, Bad, Harakiri).

Non-limiting examples of pro-apoptosis agents contemplated within the scope of the present invention include gramicidin, magainin, mellitin, defensin, cecropin, $(KLAKLAK)_2$ (SEQ ID NO:1), $(KLAKKLA)_2$ (SEQ ID NO:2), $(KAAKKAA)_2$ (SEQ ID NO:3) or $(KLGKKLG)_3$ (SEQ ID NO:4).

Angiogenic inhibitors

In certain embodiments the present invention may concern administration of targeting peptides attached to anti-angiogenic agents, such as angiotensin, laminin peptides, fibronectin peptides, plasminogen activator inhibitors, tissue metalloproteinase inhibitors, interferons, interleukin 12, platelet factor 4, IP-10, Gro-ß,

thrombospondin, 2-methoxyoestradiol, proliferin-related protein, carboxiamidotriazole, CM101, Marimastat, pentosan polysulphate, angiopoietin 2 (Regeneron), interferon-alpha, herbimycin A, PNU145156E, 16K prolactin fragment, Linomide, thalidomide, pentoxifylline, genistein, TNP-470, endostatin, paclitaxel, accutin, angiostatin, cidofovir, vincristine, bleomycin, AGM-1470, platelet factor 4 or minocycline.

Proteins and Peptides

In certain embodiments, the present invention concerns novel compositions comprising at least one protein or peptide. As used herein, a protein or peptide generally refers, but is not limited to, a protein of greater than about 200 amino acids, up to a full length sequence translated from a gene; a polypeptide of greater than about 100 amino acids; and/or a peptide of from about 3 to about 100 amino acids. For convenience, the terms "protein," "polypeptide" and "peptide are used interchangeably herein.

In certain embodiments the size of the at least one protein or peptide may comprise, but is not limited to, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, about 110, about 120, aboout 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 275, about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 475, about 500, about 525, about 550, about 575, about 600, about 625, about 650, about 675, about 700, about 725, about 750, about 775, about 800, about 825, about 850, about 875, about 900, about 925, about 950, about 975, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1750, about 2000, about 2250, about 2500 or greater amino acid residues.

As used herein, an "amino acid residue" refers to any naturally occuring amino acid, any amino acid derivitive or any amino acid mimic known in the art. In certain

embodiments, the residues of the protein or peptide are sequential, without any non-amino acid interrupting the sequence of amino acid residues. In other embodiments, the sequence may comprise one or more non-amino acid moieties. In particular embodiments, the sequence of residues of the protein or peptide may be interrupted by one or more non-amino acid moieties.

Accordingly, the term "protein or peptide" encompasses amino acid sequences comprising at least one of the 20 common amino acids found in naturally occurring proteins, or at least one modified or unusual amino acid, including but not limited to those shown on Table 2 below.

Table 2

Modified and Unusual Amino Acids

Abbr.	Amino Acid	Abbr.	Amino Acid
Aad	2-Aminoadipic acid	EtAsn	N-Ethylasparagine
Baad	3- Aminoadipic acid	Hyl	Hydroxylysine
Bala	β-alanine, β-Amino-propionic acid	AHyl	allo-Hydroxylysine
Abu	2-Aminobutyric acid	3Hyp	3-Hydroxyproline
4Abu	4- Aminobutyric acid, piperidinic acid	4Hyp	4-Hydroxyproline
Acp	6-Aminocaproic acid	Ide	Isodesmosine
Ahe	2-Aminoheptanoic acid	Alle	allo-Isoleucine
Aib	2-Aminoisobutyric acid	MeGly	N-Methylglycine, sarcosine
Baib	3-Aminoisobutyric acid	Melle	N-Methylisoleucine
Apm	2-Aminopimelic acid	MeLys	6-N-Methyllysine
Dbu	2,4-Diaminobutyric acid	MeVal	N-Methylvaline
Des	Desmosine	Nva	Norvaline
Dpm	2,2'-Diaminopimelic acid	Nle	Norleucine
Dpr	2,3-Diaminopropionic acid	Orn	Ornithine
EtGly	N-Ethylglycine		

Proteins or peptides may be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, the isolation of proteins or peptides from natural sources, or the chemical synthesis of proteins or peptides. The nucleotide and protein, polypeptide and peptide sequences corresponding to various genes have been previously disclosed, and may be found at computerized databases known to those of ordinary skill in the art. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases (http://www.ncbi.nlm.nih.gov/). The coding regions for known genes may be amplified and/or expressed using the techniques disclosed herein or as would be know to those of ordinary skill in the art. Alternatively, various commercial preparations of proteins, polypeptides and peptides are known to those of skill in the art.

Peptide mimetics

Another embodiment for the preparation of polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson *et al.*, "Peptide Turn Mimetics" in *BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, Eds., Chapman and Hall, New York (1993), incorporated herein by reference. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. These principles may be used to engineer second generation molecules having many of the natural properties of the targeting peptides disclosed herein, but with altered and even improved characteristics.

Fusion proteins

Other embodiments of the present invention concern fusion proteins. These molecules generally have all or a substantial portion of a targeting peptide, linked at the N- or C-terminus, to all or a portion of a second polypeptide or proteion. For example,

fusions may employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions. In preferred embodiments, the fusion proteins of the instant invention comprise a targeting peptide linked to a therapeutic protein or peptide. Examples of proteins or peptides that may be incorporated into a fusion protein include cytostatic proteins, cytocidal proteins, pro-apoptosis agents, anti-angiogenic agents, hormones, cytokines, growth factors, peptide drugs, antibodies, Fab fragments antibodies, antigens, receptor proteins, enzymes, lectins, MHC proteins, cell adhesion proteins and binding proteins. These examples are not meant to be limiting and it is contemplated that within the scope of the present invention virtually and protein or peptide could be incorporated into a fusion protein comprising a targeting peptide. Methods of generating fusion proteins are well known to those of skill in the art. Such proteins can be produced, for example, by chemical attachment using bifunctional cross-linking reagents, by *de novo* synthesis of the complete fusion protein, or by attachment of a DNA sequence encoding the targeting peptide to a DNA sequence encoding the second peptide or protein, followed by expression of the intact fusion protein.

Protein purification

In certain embodiments a protein or peptide may be isolated or purified. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the homogenization and crude fractionation of the cells, tissue or organ to polypeptide and non-polypeptide fractions. The protein or polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, gel exclusion chromatography, polyacrylamide gel electrophoresis,

affinity chromatography, immunoaffinity chromatography and isoelectric focusing. An example of receptor protein purification by affinity chromatography is disclosed in U.S. Patent No. 5,206,347, the entire text of which is incorporated herein by reference. A particularly efficient method of purifying peptides is fast protein liquid chromatography (FPLC) or even HPLC.

A purified protein or peptide is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. An isolated or purified protein or peptide, therefore, also refers to a protein or peptide free from the environment in which it may naturally occur. Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide are known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity therein, assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification, and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification are well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like, or by heat denaturation, followed by:

centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of these and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

Affinity chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule to which it can specifically bind. This is a receptor-ligand type of interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (*e.g.*, altered pH, ionic strength, temperature, *etc.*). The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand.

Synthetic Peptides

Because of their relatively small size, the targeting peptides of the invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, (1984); Tam *et al.*, (1983); Merrifield, (1986); and Barany and Merrifield (1979), each incorporated herein by reference. Short peptide sequences, usually from about 6 up to about 35 to 50 amino acids, can be readily synthesized by such methods. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell, and cultivated under conditions suitable for expression.

Antibodies

In certain embodiments, it may be desirable to make antibodies against the identified targeting peptides or their receptors. The appropriate targeting peptide or receptor, or portions thereof, may be coupled, bonded, bound, conjugated, or chemically-linked to one or more agents via linkers, polylinkers, or derivatized amino acids. This may be performed such that a bispecific or multivalent composition or vaccine is produced. It is further envisioned that the methods used in the preparation of these compositions are familiar to those of skill in the art and should be suitable for administration to subjects, *i.e.*, pharmaceutically acceptable. Preferred agents are the carriers are keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA).

The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, $F(ab')_2$, single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. Techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

Cytokines and chemokines

In certain embodiments, it may be desirable to couple specific bioactive agents to one or more targeting peptides for targeted delivery to an organ or tissue. Such agents include, but are not limited to, cytokines, chemikines, pro-apoptosis factors and anti-angiogenic factors. The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, growth factors and traditional polypeptide hormones. Included among the cytokines are growth hormones such as growth hormone, N-methionyl growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; prostaglandin, fibroblast growth factor; prolactin; placental lactogen, OB protein; tumor necrosis factor-.alpha. and -.beta.; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-.beta.; platelet-growth factor; transforming growth factors (TGFs) such as TGF-.alpha. and TGF-.beta.; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1.alpha., IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, LIF, G-CSF, GM-CSF, M- CSF, EPO, kit-ligand or FLT-3, angiostatin, thrombospondin, endostatin, tumor necrosis factor and LT. As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

Chemokines generally act as chemoattractants to recruit immune effector cells to the site of chemokine expression. It may be advantageous to express a particular chemokine gene in combination with, for example, a cytokine gene, to enhance the recruitment of other immune system components to the site of treatment. Chemokines include, but are not limited to, RANTES, MCAF, MIP1-alpha, MIP1-Beta, and IP-10.

The skilled artisan will recognize that certain cytokines are also known to have chemoattractant effects and could also be classified under the term chemokines.

Cross-linkers

Bifunctional cross-linking reagents have been extensively used for a variety of purposes including preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligand and receptor binding sites, and structural studies. Homobifunctional reagents that carry two identical functional groups proved to be highly efficient in inducing cross-linking between identical and different macromolecules or subunits of a macromolecule, and linking of polypeptide ligands to their specific binding sites. Heterobifunctional reagents contain two different functional groups. By taking advantage of the differential reactivities of the two different functional groups, cross-linking can be controlled both selectively and sequentially. The bifunctional cross-linking reagents can be divided according to the specificity of their functional groups, *e.g.*, amino, sulfhydryl, guanidino, indole, carboxyl specific groups. Of these, reagents directed to free amino groups have become especially popular because of their commercial availability, ease of synthesis and the mild reaction conditions under which they can be applied. A majority of heterobifunctional cross-linking reagents contains a primary amine-reactive group and a thiol-reactive group.

Exemplary methods for cross-linking ligands to liposomes are described in U.S. Patent 5,603,872 and U.S. Patent 5,401,511, each specifically incorporated herein by reference in its entirety). Various ligands can be covalently bound to liposomal surfaces through the cross-linking of amine residues. Liposomes, in particular, multilamellar vesicles (MLV) or unilamellar vesicles such as microemulsified liposomes (MEL) and large unilamellar liposomes (LUVET), each containing phosphatidylethanolamine (PE), have been prepared by established procedures. The inclusion of PE in the liposome provides an active functional residue, a primary amine, on the liposomal surface for cross-linking purposes. Ligands such as epidermal growth factor (EGF) have been successfully linked with PE-liposomes. Ligands are bound covalently to discrete sites on the liposome surfaces. The number and surface density

of these sites are dictated by the liposome formulation and the liposome type. The liposomal surfaces may also have sites for non-covalent association. To form covalent conjugates of ligands and liposomes, cross-linking reagents have been studied for effectiveness and biocompatibility. Cross-linking reagents include glutaraldehyde (GAD), bifunctional oxirane (OXR), ethylene glycol diglycidyl ether (EGDE), and a water soluble carbodiimide, preferably 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Through the complex chemistry of cross-linking, linkage of the amine residues of the recognizing substance and liposomes is established.

In another example, heterobifunctional cross-linking reagents and methods of using the cross-linking reagents are described (U.S. Patent 5,889,155, incorporated herein by reference in its entirety). The cross-linking reagents combine a nucleophilic hydrazide residue with an electrophilic maleimide residue, allowing coupling in one example of aldehydes to free thiols. The cross-linking reagent can be modified to cross-link various functional groups.

Nucleic Acids

Nucleic acids according to the present invention may encode a targeting peptide, a receptor protein or a fusion protein. The nucleic acid may be derived from genomic DNA, complementary DNA (cDNA) or synthetic DNA. Where incorporation into an expression vector is desired, the nucleic acid may also comprise a natural intron or an intron derived from another gene. Such engineered molecules are sometime referred to as "mini-genes."

A "nucleic acid" as used herein includes single-stranded and double-stranded molecules, as well as DNA, RNA, chemically modified nucleic acids and nucleic acid analogs. It is contemplated that a nucleic acid within the scope of the present invention may be of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, about 110, about 120, aboout 130, about 140, about 150, about

160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 275, about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 475, about 500, about 525, about 550, about 575, about 600, about 625, about 650, about 675, about 700, about 725, about 750, about 775, about 800, about 825, about 850, about 875, about 900, about 925, about 950, about 975, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1750, about 2000, about 2250, about 2500 or greater nucleotide residues in length.

It is contemplated that targeting peptides, fusion proteins and receptors may be encoded by any nucleic acid sequence that encodes the appropriate amino acid sequence. The design and production of nucleic acids encoding a desired amino acid sequence is well known to those of skill in the art, using standardized codon tables (see Table 3 below). In preferred embodiments, the codons selected for encoding each amino acid may be modified to optimize expression of the nucleic acid in the host cell of interest. Codon preferences for various species of host cell are well known in the art.

Table 3

Amino Acid			**Codons**
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU

Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

In addition to nucleic acids encoding the desired targeting peptide, fusion protein or receptor amino acid sequence, the present invention encompasses complementary nucleic acids that hybridize under high stringency conditions with such coding nucleic acid sequences. High stringency conditions for nucleic acid hybridization are well known in the art. For example, conditions may comprise low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. It is understood that the temperature and ionic strength of a desired stringency are determined in part by the length of the particular nucleic acid(s), the length and nucleotide content of the target sequence(s), the charge composition of the nucleic acid(s), and to the presence or concentration of

formamide, tetramethylammonium chloride or other solvent(s) in a hybridization mixture.

Vectors for Cloning, Gene Transfer and Expression

In certain embodiments expression vectors are employed to express the targeting peptide or fusion protein, which can then be purified and used. In other embodiments, the expression vectors are used in gene therapy. Expression requires that appropriate signals be provided in the vectors, and which include various regulatory elements, such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are known.

Regulatory Elements

The terms "expression construct" or "expression vector" are meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid coding sequence is capable of being transcribed. In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene. The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell.

In various embodiments, the cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter, and glyceraldehyde-3-phosphate dehydrogenase promoter can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the

art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

Where a cDNA insert is employed, typically one will typically include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed, such as growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression construct is a terminator. These elements can serve to enhance message levels and to minimize read through from the construct into other sequences.

Selectable Markers

In certain embodiments of the invention, the cells containing nucleic acid constructs of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression construct. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants. For example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin, and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

Delivery of Expression Vectors

There are a number of ways in which expression vectors may be introduced into cells. In certain embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome, and express viral genes stably and efficiently have made them attractive

candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). Preferred gene therapy vectors are generally viral vectors.

Although some viruses that can accept foreign genetic material are limited in the number of nucleotides they can accommodate and in the range of cells they infect, these viruses have been demonstrated to successfully effect gene expression. However, adenoviruses do not integrate their genetic material into the host genome and therefore do not require host replication for gene expression, making them ideally suited for rapid, efficient, heterologoous gene expression. Techniques for preparing therapeutic viruses are well known in the art.

In using viral delivery systems, one will desire to purify the virion sufficiently to render it essentially free of undesirable contaminants, such as defective interfering viral particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the cell, tissue or intact organism receiving the vector construct. A preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

Viruses used as gene vectors were DNA viruses may include the papovaviruses (*e.g.*, simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986).

One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide that has been cloned therein.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retroviral infection, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5′-tripartite leader (TPL) sequence which makes them preferred mRNAs for translation.

In currently used systems, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3, or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Racher *et al.*, (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by innoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) are employed as follows. A cell innoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking is initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking is commenced for another 72 hr.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to

be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

A typical vector applicable to practicing the present invention is replication defective and will not have an adenovirus E1 region. Thus, it is most convenient to introduce the polynucleotide encoding the gene at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.*, (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1991). Animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et*

al., 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic innoculation into the brain (Le Gal La Salle *et al.*, 1993).

Other gene transfer vectors may be constructed from retroviruses. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, *gag*, *pol*, and *env* that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences, and also are required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a protein of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol*, and *env* genes, but without the LTR and packaging components, is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are capable of infecting a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

There are certain limitations to the use of retrovirus vectors. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This may result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the *gag, pol, env* sequence integrated in the host cell genome. However, packaging cell lines are available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

Other viral vectors may be employed as expression constructs. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988), adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984), and herpes viruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990), DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection, DNA-loaded liposomes and lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar

liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers. Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Nicolau *et al.*, (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

A number of selection systems may be used to identify or select for transformed cells, including, but not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in *tk*-, *hgprt*- or *aprt*- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr:* that confers resistance to methotrexate; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

Pharmaceutical compositions

Where clinical applications are contemplated, it may be necessary to prepare pharmaceutical compositions - expression vectors, virus stocks, proteins, antibodies and drugs - in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of impurities that could be harmful to subjects.

One generally will desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also are employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention may comprise an effective amount of a protein or peptide, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such

compositions also are referred to as innocula. The phrase "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to a subject. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the proteins or peptides of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions may occur via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions normally would be administered as pharmaceutically acceptable compositions, described *supra*.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid,

thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Dosages

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, and in particular to pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by the FDA Office of Biologics standards.

EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific

embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1: Re-targeting of Gene Therapy Vectors Using Bifunctional Conjugates

This example demonstrates the feasibility of using organ-homing peptides to target adenoviral or other vectors to receptors specifically expressed in the endothelia of certain organs. A bispecific conjugate was designed, consisting of one part that bound to the Ad5 phage and another part that was a targeting peptide selective for a specific organ or tissue. A bispecific adaptor was chosen because direct chemical conjugation of homing peptides onto the virus capsid abolished the infectivity of Ad5 vectors (data not shown). Further, cloning targeting peptides into the capsid genome could possibly change the specificity of the ligand through conformational changes of the peptide structure. Moreover, previous attempts to genetically modify the virus capsid did not abolish natural tropism because the amino acid sequence of the fiber knob protein that mediates the CAR interaction could not be deleted or mutated (Michael *et al.*, 1995; Wickham *et al.*, 1995; Wickham *et al. 1997b*; Dmitriev *et al.*, 1998, Vigne *et al.*, 1999).

In this example, it was demonstrated that Fab fragments of an anti-Ad5 antibody conjugated to the GFE-1 lung-homing peptide (Rajotte *et al.*, 1998; Rajotte and Ruoslahti, 1999) could be used to successfully re-target Ad5 vectors. Cell types that were not normally susceptible to Ad5 infection but that expressed the receptor for the GFE-1 peptide were efficiently transduced in the presence of the bispecific adaptor conjugate. This was the first demonstration of a functional Fab–targeting peptide conjugate. Such molecular adaptors may be of use for systemic delivery of Ad5 gene-therapy vectors to receptors specifically expressed in the vasculature of certain organs or tissues, such as tumor tissue. Although the preferred embodiment disclosed in the present example utilized an adeniviral vector, the skilled artisan will realize that the disclosed methods could be used with virtually any gene therapy vector.

Materials and Methods

Virus production and infection for immunofluorescence or immunoprecipitation

HeLa cervical carcinoma cells and 293 embryonic kidney cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. Wild-type Ad5 and E1-deleted recombinant viruses expressing as maker genes either the *Aequorea victoria* green fluorescent protein (Ad5-GFP) or the *Escherichia coli* LacZ gene (Ad5-LacZ) under the control of the CMV promoter were propagated in 293 cells. Viruses were harvested from cell pellets and purified by sequential rounds of ultracentrifugation in CsCl gradients according to standard protocols known in the art. Virus concentrations were estimated by measuring absorbance at A_{260} (1 absorbance unit corresponds to approximately 10^{12} virions), and infectious titers were determined by plaque assays. For infections preceding immunofluorescence or immunoprecipitation, cells were incubated with Ad5 at an MOI of 10 or 50 PFU/cell in DMEM containing 2% FBS. After 5 h, fresh FBS was added to 10% and infections were allowed to proceed for 24 h.

Monoclonal antibody production

Female 2-month-old Balb/C mice (Harlan Sprague Dawley) were given intraperitoneal (i.p.) injections of 10^9 particles of wild-type Ad5 at 2-week intervals over a period of 2 months. After the third and fourth immunization, the serum of each immunized mouse was analyzed by ELISA. The spleen from the mouse with the highest anti-Ad5 antibody titer was removed, and splenocytes were isolated and fused in a 1:5 ratio with Sp2OAG14 mouse myeloma cells (ATCC). Fused cells were plated in 96-well plates without feeder layer cells. Hybridoma clones were selected, expanded, and subcloned in DMEM containing 10% CPSR (controlled process serum replacement), 1x HAT, 10% Hybrimax hybridoma medium supplement, and 1% penicillin/streptomycin (all from Sigma). Single clones were obtained by limiting dilution. Antibody production was monitored by the ELISA technique on Ad5 antigen using hybridoma supernatants.

To obtain larger amounts of antibodies, female 3-month-old Balb/C nude mice (Harlan Sprague Dawley) were primed with pristane for ascites production and injected

i.p. with 2 x 10^6 hybridoma cells per mouse (Harlow and Lane, 1988, the entire text of which is incorporated herein by reference). Ascites fluid was harvested after 14–29 days. Antibodies were purified from ascites with protein G-sepharose (GammaBind, Pharmacia) according to the manufacturer's instructions. The monoclonal antibodies described in this example were derived from two separate immunization and fusion experiments.

ELISA

Wild-type Ad5 virions or recombinant fiber knob protein (Krasnykh *et al.*, 1996) were immobilized in PBS (10^9 particles or 5 μg/well) on High Binding Assay Plates (Costar). Control wells were coated with 2 mg bovine serum albumin (BSA) in PBS overnight at 4 °C. Primary antibodies or control polyclonal mouse IgG (Sigma) were then incubated at a range of concentrations for 1 h at room temperature. The secondary antibody (anti-mouse-Fab alkaline phosphatase-conjugate, Sigma, 1:3000 in 3% BSA) was added and incubated for 1 h. The ELISA was developed with p-nitrophenyl phosphate (Sigma), and readings were taken 1–4 h later at 405 nm (Reader 520, Organon Teknika).

Immunofluorescence staining

HeLa cells grown on coverslips were infected at an MOI of 50 PFU/cell. At 24 h after infection, cells were washed in PBS and fixed in 3.7% formaldehyde, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS, washed again with PBS, and blocked with 3% BSA in PBS for 20 min. The mouse monoclonal antibodies were added to slides at 10 μg/ml for 1 h at room temperature in 3% BSA, and a rabbit anti-Ad5 polyclonal antibody (Kozarsky *et al.*, 1996) was used at a dilution of 1:1000. Cells were washed and incubated for 1 h with a fluorescein-coupled secondary antibody (1:200, Jackson Laboratories). Samples were washed in PBS and incubated for 5 min in 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS. Coverslips were mounted with Fluoromount-G (Southern Biotechnology Associates) and staining was visualized by epifluorescence using a Nikon microscope in conjunction with a CCD camera (Cooke

Sensicam). Images were obtained in double excitation mode and processed with SlideBook and Adobe Photoshop.

Immunoprecipitation and Western blot analysis

Cells were lysed in 50 mM Tris-HCl pH 7.6, 1% NP-40, 150 mM NaCl, and 0.1 mM ZnOAc in the presence of protease inhibitors. Protein concentration was determined by the Lowry method (Bio-Rad). Proteins were immunoprecipitated from 1 mg of cell extracts with protein G-sepharose (Pharmacia) and 5 μg/ml of monoclonal antibodies. Alternatively, immunoprecipitations were performed from purified virus stocks by using 3 μg of CsCl-purified Ad5. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, blotted with a rabbit polyclonal anti-Ad5 antibody (Kozarsky *et al.*, 1996) and anti-rabbit IgG HRP (Jackson Laboratories), and visualized by enhanced chemiluminescence (Renaissance, NEN).

Ad5 neutralization assay

HeLa cells were plated at a density of 20,000 cells/well in a 96-well plate 24 h before use. Antibodies were diluted in DMEM and incubated for 1 h at 37 °C with Ad5-LacZ at a concentration of $2x10^7$ PFU/ml. Subsequently, the antibody-Ad5-LacZ complexes were incubated for 90 min on HeLa cells at an MOI of 50 PFU/cell, followed by the addition of 1.8 volumes of DMEM containing 10% FBS. After 24 h, cells were fixed in 4% paraformaldehyde and stained for ß-galactosidase expression (In-Situ-ß-Galactosidase Staining Kit, Stratagene). At least 150 cells per well were counted and used to calculate the percentage of ß-galactosidase-positive cells.

Phage binding assays on cells

RD human rhabdomyosarcoma cells (ATCC) and MDA-MB-435 human breast cancer cells (Price *et al.*, 1990) were grown according to standard procedures in DMEM containing 10% FBS, and 1% streptomycin/penicillin. Phage binding assays to cells were performed with the GFE-1 phage, a fUSE5-based phage clone that displayed the

sequence CGFECVRQCPERC (SEQ ID NO:5). The insertless fd-tet phage was used as a control. RD or MDA-MB-435 cells were grown in 24-well plates to a density of 300,000 cells per well. Medium was removed and replaced with complete medium containing 2 x 10^8 transducing units of either GFE-1 or fd-tet phage. Cells were incubated with the phage particles for 3 h at 4 °C and then washed four times with 1 ml DMEM containing 10% FBS. Bound phages were rescued by adding 1 ml of a K91Kan terrific broth culture. Multiple dilutions from this culture were plated on LB agar plates containing 20 μg/ml tetracycline and grown at 37 °C for 16 h before colonies (transducing units) were counted.

Production of antibody-peptide conjugates

Purified 1C5IIE11 anti-Ad5 IgG was digested for 9 h with immobilized papain in papain digestion buffer (both from Pierce) containing 0.05% Tween20. The digestion mix was then passed over a GammaBind protein G column (Pharmacia) equilibrated with borax buffer pH 8.0 containing 0.05% Tween20. The column flow-through was slowly concentrated to 5 mg/ml (Centricon tubes, Amicon, molecular weight cut-off 10,000). The purity of the obtained Fab fragments was verified by SDS-PAGE and Coomassie Blue staining.

The GFE-1 peptide (CGFECVRQCPERC, SEQ ID NO:5) and a cyclic control peptide (CARAC, SEQ ID NO:6) were synthesized and purified by Anaspec. The purified Fab was added to a 20-fold molar excess of either peptide and the homobifunctional crosslinker bis(sulfosuccinimidyl)suberate (BS^3, Pierce). After 2 h of incubation at room temperature, the products of the crosslinking reaction were purified by dialysis against 1000 volumes of borax buffer pH 8.5 containing 0.05% Tween20. Complete removal of the detergent from the solution resulted in precipitation and concentration loss of the sample. Despite several attempts, this reaction prevented measurement of mass of the conjugate by MALDI-TOF mass spectrometry. Successful conjugation was determined instead by SDS-PAGE and Coomassie Blue staining with a 4–20% gradient Tris-glycine gel (Novex). The antigen binding activity of Fab and its conjugates was verified by ELISA as described above.

Membrane dipeptidase assay

Evaluation of membrane dipeptidase activity was performed by fluorometric detection of D-Phe in cell lysates in presence and absence of the Fab-GFE adaptor. 10^6 RD cells were lysed in 100 µl of TBS/100 mM octyl glycoside without protease inhibitors. Protein concentration of the lysates was determined by the Lowry method (Bio-Rad). Lysates normalized to 10 µg protein were first incubated for 15 min at 37°C in 0.1 M Tris-HCl pH 8 containing a range of concentrations of the Fab-GFE adaptor or vehicle, respectively. Enzyme activity was then detected as disclosed (Heywood and Hooper, 1995). Briefly, samples were prepared in triplicate and incubated at 37°C for 3 h with the MDP substrate Gly-D-Phe at 1 mM. The released D-Phe was then detected indirectly by converting it to the fluorescent dye 6,6'-dihydroxy-(1,1'-biphenyl)3,3'-diacetic acid in the presence of D-amino acid oxidase and peroxidase (all from Sigma). Fluorescence was detected using an f_{max} fluorescence microplate reader (Molecular Devices, CA), using excitation wave length 317 nm, emission wave length 414 nm, slit width = 5 nm, integration time = 1 s.

Ad5 re-targeting assay

RD or MDA-MB-435 cells were seeded at a density of 20,000 cells/well in a 96-well plate 24 h before infection. Fab fragments or Fab-peptide conjugates were diluted in DMEM and incubated for 1 h at 37°C with 6×10^7 particles/ml Ad5-LacZ. The Fab-Ad5-LacZ complexes were then incubated for 90 min with HeLa cells at an MOI of 150 PFU/cell. After 90 min, the supernatant was removed and replaced with DMEM containing 10% FBS. After 24 h, cells were fixed in 4% paraformaldehyde and stained for ß-galactosidase expression (In-Situ ß-Galactosidase Staining Kit, Stratagene). At least 150 cells per well were counted and used to calculate the percentage of ß-galactosidase-positive cells. The procedure for targeting Ad5-GFP vectors was the same as that for the Ad5-LacZ vectors except that GFP-expressing cells were visualized not by staining but rather by epifluorescence with a Nikon Eclipse TE300 microscope in conjunction with a SPOT Imaging System. In addition, cells infected with Ad5-GFP on 24-well plates were detached with 2.5 mM EDTA 24 h after infection and fluorescent

cells were counted in a FACSORT flow cytometer (Becton Dickinson, Germany). Data were analyzed with Cell Quest.

Results

Generation and characterization of anti-Ad5 monoclonal antibodies

Anti-Ad5 monoclonal antibodies were generated from mouse splenocytes. Hybridoma clones that stably expressed anti-Ad5 monoclonal IgGs were initially characterized by ELISA using intact Ad5 virus particles (data not shown). Indirect immunofluorescence of Ad5-infected cells confirmed that the antibodies recognized adenovirus proteins. Therefore, HeLa cells were infected with Ad5 at a multiplicity of infection (MOI) of 50 plaque-forming units (PFU)/cell, and the six monoclonal antibodies were used for staining. A rabbit polyclonal antibody serum against Ad5 was used as a control, and produced a strong signal for infected cells (not shown). The 1C5IIE11 and 3B2ID10 antibodies produced a similar fluorescent signal in the nucleus of infected cells. No signal was detected in uninfected cells or with secondary antibody alone.

Antibodies were also tested for their ability to precipitate adenoviral proteins from infected 293 cells. Immunoprecipitates from uninfected cell extracts were negative. In infected cells, the most prominent band, at approximately 60 kDa, represented the fiber protein. Another prominent band at approximately 70 kDa represented the penton base. One antibody also precipitated a third band at 120 kDa, corresponding to the viral hexon protein (data not shown). These 3 proteins were also detected by the control polyclonal antibody in western blots of extracts from Ad5-infected 293 cells (data not shown). Immunoprecipitations from purified Ad5 particles revealed similar results (data not shown), verifying that the antibodies recognize fully assembled, purified adenoviral capsids.

Of the three major proteins in the Ad5 capsid (i.e., the hexon, penton base, and fiber, the latter composed of the shaft and knob), the fiber knob region is a suitable binding site for a re-targeting adaptor because that region mediates attachment of the

virus to the target cell (Henry *et al.*, 1994; Louis *et al.*, 1994). Thus, an adaptor bound to the fiber knob could also neutralize the endogenous tropism of Ad5-based vectors. ELISA of immobilized recombinant fiber knob protein showed that two of the antibody clones, 3B2ID10 and 1C5IIE11, bound the fiber knob protein of the Ad5 capsid.

Neutralization of Ad5 infection

Because ablation of the natural tropism of the virus is desirable for re-targeting strategies, the ability of the anti-Ad5 antibodies and Ad5-binding peptides to neutralize recombinant Ad5 infection was tested. HeLa cells were infected with recombinant Ad5-LacZ vectors in the presence and absence of antibodies. The 1C5IIE11 and the 3B2ID10 antibodies showed significant neutralization activity, with the other four antibodies showing weaker but reproducible neutralization of Ad5 infection (FIG. 1).

Characterizing cell lines suitable for re-targeting Ad5 transduction

To define suitable models for vector targeting in vitro, cell lines were characterized based on two parameters: (1) the lack of permissivity to Ad5 infection; and (2) expression of the receptor for the GFE-1 targeting peptide. Two cell lines, RD and MDA-MB-435, were tested for Ad5 susceptibility by incubating with recombinant Ad5-LacZ at MOIs of 25, 50, 100, and 200 PFU/cell, and ß-galactosidase expression was determined 24 h after infection. Neither cell line expressed this marker at MOIs of 100 or less. At an MOI of 200, a small fraction of cells (2–6% RD, 0–3% MDA-MB-435) showed blue staining (data not shown).

Next, it was determined whether these cells express membrane dipeptidase (MDP), the receptor for the lung-targeting peptide GFE-1 (Rajotte et al, 1998; Rajotte and Ruoslahti, 1999). Because antibodies against human MDP are not available, cell binding of a phage clone displaying GFE-1 was compared with that of the wild-type, insertless fd-tet phage. The GFE-1 phage bound both cell lines more than 20 times more strongly than did the fd-tet phage (FIG. 2). These findings suggest that RD and

MDA-MB-435 cells express the GFE-1 receptor and are suitable for re-targeting Ad5 vectors with adaptors containing GFE-1 as the targeting moiety.

Generation of GFE-based molecular adaptors

One of the monoclonal antibodies that could neutralize Ad5 infection was used to generate a molecular adaptor for Ad5 delivery via the GFE-1 receptor. Of the six anti-Ad5 antibodies described above, 1C5IIE11 was deemed the most suitable, as it showed the greatest binding strength and specificity.

Standard techniques for generating bispecific conjugates, such as those with the heterobifunctional crosslinker SPDP (N-succinimidyl[pyridyldithio]propionate) or carbodiimide derivatives, are unsuitable for linking cyclic peptides to Fab fragments, either because they require chemical reduction, which would destroy disulfide bridges within the peptides, or because the crosslinker does not provide the spacer arm necessary for linking relatively small functional moieties to Fab fragments. A conjugation protocol was established involving the homobifunctional crosslinker bis(sulfosuccinimidyl)suberate (BS^3), which proved to be suitable for linking cyclic oligopeptides to Fab fragments of mouse monoclonal antibodies.

Fab fragments of the 1C5IIE11 antibody were generated by papain digestion and crosslinked to either the GFE-1 peptide or a control peptide (CARAC, SEQ ID NO:6). The products of the conjugation reaction were analyzed by SDS-PAGE and Coomassie blue staining. The unconjugated Fab showed a clear-cut band at approximately 50 kDa, and the conjugates (Fab-GFE and Fab-CARAC) showed a "smear" reaching from 50 to about 65 kDa, suggesting that multiple copies (approx. 5-10) of a given peptide were conjugated to each Fab fragment (data not shown).

The functionality of the two binding moieties of the bispecific Fab-GFE conjugate was examined. ELISA verified that the unconjugated Fab and the Fab-peptide adaptors bound Ad5 equally well (data not shown). Neutralization assays using the intact 1C5IIE11 antibody, 1C5IIE11 derived Fab and Fab-GFE proved that the digestion and conjugation did not affect the antibody's neutralization capacity (FIG. 3).

The properties of the adaptor's GFE peptide moiety were evaluated, based on the observation that the functional GFE-1 peptide inhibited membrane dipeptidase (MDP) function (Rajotte and Ruoslahti, 1999). The Fab-GFE adaptor specifically inhibited the enzymatic activity of MDP in a dose-dependent manner (FIG. 4), showing that the Fab-GFE adaptor bound to MDP, the GFE-1 peptide's receptor.

Sensitization of RD and MDA-MB-435 cells to Ad5 infection by the Fab-GFE targeting adaptor

The ability of the generated conjugates to target Ad5-based vectors to the GFE-1 receptor was evaluated. Ad5-LacZ was added and the cells staining positive for ß-galactosidase were counted 24 h after infection with the vector alone or the vector plus one of the following adaptors: a Fab fragment of 1C5IIE11 (Fab), that fragment conjugated to CARAC (SEQ ID NO:6) (Fab-CARAC), or that fragment conjugated to the GFE-1 peptide (Fab-GFE). Infection of RD cells with Ad5-LacZ alone or in combination with Fab or Fab-CARAC produced 0.2%–3.3% positive-staining cells. However, infection with the Fab-GFE conjugate produced a concentration-dependent increase in ß-galactosidase-positive RD cells, with an estimated EC_{50} value of 6 μg/ml of the Fab-GFE adaptor at an MOI of 150 PFU/cell (FIG. 5). Saturation was reached at about 50 μg/ml, which produced 95%-98% positive cells. Similar results were obtained with MDA-MB-435 cells, but transduction efficiency was somewhat lower in this cell line (maximum approximately 80% positive cells, estimated EC_{50} of 10 μg/ml; FIG. 5).

The Fab-GFE adaptor was evaluated using an Ad5 vector that carried a different reporter gene, the green fluorescent protein (Ad5-GFP). The results were qualitatively similar to those obtained with Ad5-LacZ (not shown). Flow cytometric measurements of the numbers of Ad5-GFP-infected cells with and without addition of the molecular adaptor were similar to those obtained with fluorescent microscopy and ß-galactosidase staining (data not shown).

Discussion

Ad5-based vectors are a very efficient means of transferring genes *in vitro* and *in vivo*. A major limitation of adenoviral and other gene therapy vectors is their inability to specifically transduce the cells, tissues or organs of interest. The present example shows that Ad5-based gene-transfer vectors can be re-targeted to specific cell surface receptors via CAR-independent pathways by using bispecific adaptors that contain a targeting peptide moiety attached to an AD5 fiber protein specific moiety. A novel conjugation protocol was used to generate adaptors consisting of Fab fragments of a mouse monoclonal anti-Ad5 antibody and the GFE-1 lung-homing peptide. This adaptor re-targeted adenovirus to GFE-1-peptide receptor-positive cells. This effect was not observed with anti-Ad5 Fab alone or Fab conjugated to a control peptide.

In contrast to previous gene therapy targeting strategies, the present methods utilized an organ-homing peptide as the targeting moiety of a bispecific conjugate. Although peptide-based re-targeting of adenoviruses has been reported recently (Hong *et al.*, 1999; Romanczuk *et al.*, 1999) the present approach is novel in two aspects. (1) For the first time, a receptor-specific targeting peptide was conjugated to Fab antibody fragments. (2) The targeting peptide was selected by *in vivo* phage display.

The receptors targeted by peptides that were isolated by *in vivo* screenings are not only specific to the vasculature of a particular organ, but were also selected for their accessibility to circulating ligands. These characteristics are essential for targeting systemically administered compounds. The present example shows that these receptors can serve as a target to re-direct the tropism of Ad5-based gene therapy vectors. As the skilled artisan will realize, the present methods can be utilized with virtually any gene therapy vector where a Fab antibody fragment or other binding moiety that binds to the vector can be linked to an appropriate targeting peptide. The present results may be of use for targeted gene therapy *in vivo*.

The membrane dipeptidase (MDP) receptor for the GFE-1 peptide was targeted (Rajotte and Ruoslahti, 1999). MDP is expressed on lung vascular endothelial cells. Extensive studies based on *in vivo* homing using the GFE phage as well as

immunostaining with anti-MDP antibodies clearly demonstrated that this vascular protease is not accessible to circulating ligands in other organs (reviewed in Rajotte and Ruoslahti, 1999). However, MDP was expressed on the surface of certain tumor cell lines (Rajotte and Ruoslahti, 1999). Thus, the present methods should allow targeting of tumor cells using a GFE-based adaptor, as well as targeted therapy of other tissues or organs for which selective targeting peptides are identified.

Example 2. Adenoviral targeting motifs

Several novel motifs were identified in adenoviral binding peptides. Targeting peptides binding to the Ad5 adenovirus were prepared from a CX_8C phage display library by the methods described above. The following adenoviral targeting peptides were identified. Apparent conserved motifs are underlined.

C<u>ELR</u>LNSILC	(SEQ ID NO:21)	phage 52-7
C<u>ELR</u>EQVG<u>R</u>C	(SEQ ID NO:22)	phage 52-1
CWYTE<u>GR</u>MIC	(SEQ ID NO:23)	phage 52-40
CHSLLEKGWC	(SEQ ID NO:24)	phage 52-31

Phage bearing the identified adenoviral targeting peptides exhibited substantially elevated binding to Ad5, as shown in FIG. 6. The 52-7 phage bound to Ad with the lowest affinity. The 52-1, 52-31 and 52-40 phage exhibited much higher affinity for Ad5 binding. The skilled artisan will realize that the adenoviral targeting peptides disclosed herein will be of use for the preparation of chimeric adaptors, as disclosed in Example 1, that can be used in conjunction with organ or tissue targeting peptides for the selective delivery of adenoviral gene therapy vectors to desired organs or tissues.

Example 3. Construction of bispecific targeting peptides against adenovirus, adeno-associated virus (AAV) and tumor

Example 1 demonstrated that bispecific adaptors can be used to target adenovirus to specific organs or tissues, such as the lung. In that instance, the bispecific adaptor comprised a Fab fragment of an anti-adenovirus antibody linked to a lung targeting peptide. It is also possible to develop bispecific targeting peptides, with one portion of the peptide directed against a therapeutic agent such as a gene therapy vector and the other portion targeted against a specific organ or tissue. In order to isolate adaptor peptides with dual specificities (chimeric peptides containing a tumor vasculature-homing moiety and an adenovirus or AAV-binding moiety), a chimeric random library was designed and screened in which phage displayed the CNGRC tumor vasculature-homing peptide linked to a randomized six amino acid insert. The library structure can be represented as $CNGRCX_6$, where C is a cysteine and X is any amino acid. In these libraries, angiogenic vasculature-homing sequences are represented along with a random peptide insert that can be displayed in different contexts, which allows multiple folding arrangements. The libraries featured the tumor-homing peptide in a cyclic configuration, because higher binding affinities are achieved with cyclic peptides (Koivunen et al., 1995; Pasqualini et al., 1995).

An aliquot of the library containing 2.5×10^{12} transducing units (TU) was used in the first round of panning. The screening was performed with adenovirus particles coated on microtiter wells. Three rounds of panning were used for phage enrichment. To rescue phage bound to adenovirus, the wells were directly infected with bacteria. Recovered phage were sequenced from randomly selected clones after three rounds of panning as described (Koivunen et al., 1995; Pasqualini et al., 1995). Several distinct CNGRC-containing sequences that interact with adenovirus were recovered (Table 4).

Two of the CNGRC-containing peptides selected on adenovirus, CNGRCRLASSA (SEQ ID NO:9) and CNGRCTMGVRA (SEQ ID NO:12), are of particular interest because they appeared more frequently among the clones analyzed during the screening. The preponderance of a given clone often indicates its specificity.

Table 4. Sequences displayed by phage binding to adenovirus selected from the CNGRC-X_6 library.

Peptide Motif	% Phage Sequenced
CNGRCRLASSA (SEQ ID NO:9)	15%
CNGRCRLDYRF (SEQ ID NO:10)	8%
CNGRCSRSGPA (SEQ ID NO:11)	6%
Other frequently appearying motifs	
CNGRCTMGVRA (SEQ ID NO:12)	
CNGRCTSGRVG (SEQ ID NO:13)	
CNGRCTSGPGW (SEQ ID NO:14)	

The most promising CNGRC-adenovirus binding peptide adaptors were injected in tumor-bearing mice to demonstrate that the CNGRC peptide, when displayed in the context of the virus binding peptide, could still home into tumors. There was no detectable decrease in the tumor-homing ratios obtained with CNGRCRLASSA (SEQ ID NO:9) phage and the original CNGRC-containing phage recovered in tumor screenings (not shown).

These results demonstrate that it is possible to derive bispecific homing peptides, containing one moiety that binds to a therapeutic agent such as an adenovirus or other gene therapy vector and another moiety that binds to an organ or tissue localliz ed receptor. This allows the targeted delivery of therapeutic agents to specific organs or tissues using a single targeting peptide. This approach is advantageous in that

it eliminates the need to obtain a separate Fab fragment or other molecule targeted against the therapeutic agent, as well as the need to cross-link the a Fab fragment to an organ targeting peptide. The skilled artisan will realize that this approach is not limited to targeted delivery of adenovirus or even of gene therapy vectors in general, but rather may be utilized for any therapeutic agent that it is desired to deliver to an organ, tissue or cell type.

Example 4. Panning of random phage display peptide libraries on adenovirus (Ad5)

Additional targeting peptides of use in binding to adenoviral or AAV delivery vectors were isolated. A variety of phage libraries, listed in Table 5 below, were screened with immobilized viral particles. Immobilized BSA, casein, or gamma globulin were used as negative controls. After three rounds of panning, phage binding preferentially to adenovirus or to AAV were selected from the libraries based on a marked enrichment (approximately 20-fold) relative to the control proteins. Following the third round of selection, sequencing of the inserts from the individual phage selected on adenovirus revealed a number of peptide motifs.

Phage were sequenced from randomly selected clones after three rounds of panning as described (Koivunen et al., 1995; Pasqualini et al., 1995). Phage displaying peptides that interacted specifically with adenovirus were isolated from multiple libraries (Table 5). The specificity of each phage was assessed in binding assays that used individually amplified phage (Koivunen et al., 1995). Briefly, microtiter plate wells were coated with 10^{10} pfu of adenovirus (serotype 5 = Ad5) or 1.5 mg casein (control protein). Phage were allowed to bind for 1h, followed by vigorous washing procedures. Bound phage were then rescued by direct incubation with K91 kan bacteria for 1 hr. Enrichment was assessed by plating the infected bacteria at various concentrations on tet-plates and counting colonies after overnight growth.

Table 5. Sequences displayed by phage binding to adenovirus.

Peptide Motif	Library
SWYSQF (SEQ ID NO:15)	X_6
AVSECF (SEQ ID NO:16)	X_6
KECQSRLSCP (SEQ ID NO:17)	X_2XC_5CX
CEFRLNSILC (SEQ ID NO:18)	CX_9
CHSLLEKGWC (SEQ ID NO:19)	CX_9
CTRSFARKEC (SEQ ID NO:20)	CX_9

The adenoviral targeting peptides identified above may be used to generate bispecific molecules that can target a therapeutic adenovirus to specific organs or tissues. Using the methods discussed in Example 4 above it is possible, for example, to screen chimeric libraries comprising the virus-binding peptides in combination with a random peptide insert for binding *in vivo* to tumor-bearing mice. Phage isolated from the tumors should contain peptide inserts with both tumor-targeting and adenoviral binding properties. Alternatively, using standard cross-linking methods, it is possible to construct bispecific targeting peptides containing both a viral binding sequence and a known targeting peptide against any organ or tissue.

Panning of random phage display peptide libraries on Adeno-associated virus (AAV)

A similar strategy was used to determine the specificity of phage isolated in screenings using AAV. Microtiter plate wells were coated with $5x10^9$ units AAV or 1.5 mg control protein (for round I BSA, round II casein, round III gamma-globulins, round IV BSA, round V casein). The library was allowed for bind for 1 hr, followed by vigorous washing procedures. Protein bound phage were then rescued by direct incubation with K91 kan bacteria for 1 hr. Enrichment was assessed by plating the

phage infected bacteria at various concentrations on tet-plates and counting colonies after overnight growth. Rescued phage were bulk amplified overnight and purified. The following round of selection was performed using 10^9 transducing units per coated microtiter well. This approach has been successfully used to isolate targeting peptides selectively binding AAV.

Example 5: Modulation of the Humoral Immune Response by Lymph Node Targeting

Modulation of the immune system response to an immunogenic virus was performed using targeting peptides that directed the virus to lymph nodes of the host organism.

Materials and Methods

In vivo phage display

In vivo phage display was used to select targeting peptides that home to the vascular endothelium of lymph nodes in mice. A total of 10^7 transducing units of a random phage display library with the general peptide insert X_2CX_4CX (C = cysteine, X = any residue) were injected into the tail vein of female 2-month old nude Balb/c mice under deep anesthesia. Five minutes after injection, the mice were euthanized by perfusion of 5 ml of DMEM through the heart.

To recover bound phage, the axillary lymph nodes and control organs (brains and pancreas) were surgically removed, weighed and ground with a glass Dounce homogenizer in one ml of DMEM plus protease inhibitors (1 mM PMSF, 20 μg/ml aprotinin, 1 μg/mol leupeptin). The tissues were washed three times with 1 ml of ice-cold washing media (DMEM-PI plus 1% BSA). After 3 washes, the tissues were incubated with 1 ml of starved competent *E. coli* K91kan and serial dilutions of the bacterial cultures were spread onto LB agar plates containing 40 μg/ml of tetracycline and 100 μg/ml of kanamycin. Standard phage amplification, purification, and selection of individual clones were performed (Pasqualini *et al.*, 2000a). In brief, three rounds of selection were performed pooling 10^3 individual colonies obtained from the first round. Single colonies were grown separately for 12 hours in 5 ml of NZY medium containing

40 μg/ml of tetracycline. Bacterial cultures were pooled, the phage preparations were purified, and 10^9 T.U. were re-injected into mice.

Validation of lymph node targeting.

Phage displaying motifs and/or peptides that were isolated multiple times in successive rounds were used for further analysis to determine their selectivity for lymph node. The number of phage T.U. bearing the selected sequence recovered from lymph node was compared to the number of phage T.U. recovered from brain and pancreas control organs (normalized by mass). Lymph node-homing phage T.U. counts were also compared to either insertless control phage or unselected X_2CX_4CX phage library. To evaluate homing, four axillary lymph nodes were harvested in each experiment.

Two phage clones displaying the peptides PTCAYGWCA (SEQ ID NO:7) and WSCARPLCG (SEQ ID NO:8) yielded the best lymph node/control ratios. Other phage displaying peptides with the motifs CAY and SCAR (data not shown) were also recovered from the lymph nodes during multiple rounds of *in vivo* selection.

Phage clones displaying the PTCAYGWCA (SEQ ID NO:7) and WSCARPLCG (SEQ ID NO:8) sequences were compared for lymph node homing to unselected phage library or insertless phage as negative controls. Individual phage clones were injected intravenously into female 2-month old nude Balb/c mice and phage were recovered as described above. To confirm specificity and to show that the displayed peptides mediated homing to lymph nodes, the cognate soluble peptides PTCAYGWCA (SEQ ID NO:7) and WSCARPLCG (SEQ ID NO:8) were synthesized, purified, cyclized (Anaspec, CA), and tested for the ability to inhibit phage homing. Competition of phage homing with the cognate peptide *in vivo* was performed by co-administration of 1 mg of each of the synthetic peptides per experiment.

Modulation of immunogenic response using lymph node targeting peptides

The host immune response to phage displaying the lymph node-homing peptides PTCAYGWCA (SEQ ID NO:7) or WSCARPLCG (SEQ ID NO:8) was compared to that produced in response to insertless control phage (fd-tet phage). Phage (from 10^6 to 10^8 T.U.) were injected into the tail veins of female 2-month old Balb/c immunocompetent mice. The phage batches were prepared simultaneously and endotoxins were removed from the preparations prior to vaccination. Each experiment

was performed using an independent preparation of each of the lymph node-homing phage clones and the negative control phage (fd-tet). Two mice were injected per phage sample, with boosting at two-week intervals. The mice were bled six days after the first vaccination and three days after the second and third vaccinations to assess immune response. A total of 234 vaccinations were performed in 78 Balb/c mice in three independent cohorts. Anti-phage antibody serum titers were determined by ELISA by using immobilized phage particles (fd-tet, 10^5 T.U. per 96-microtiter well). Serum dilutions were 1:500 and plates were coated overnight at 4°C. Data represent optical density values (OD_{450}) of the p-nitrophenyl phosphate substrate after two immunizations. Additional sets of mice vaccinated with either PTCAYGWCA (SEQ ID NO:7) or WSCARPLCG (SEQ ID NO:8) phage were injected with 1 mg of the cognate peptide prior to phage vaccination.

Results

Two peptides that mediated homing of phage to lymph nodes upon systemic administration were isolated. The sequences of these peptides were PTCAYGWCA (SEQ ID NO:7) and WSCARPLCG (SEQ ID NO:8), with CAY and SCAR representing motifs that also occurred in other phage clones targeting lymph node tissue. In contrast to a phage lacking a peptide insert (fd-tet phage), phage displaying the peptides PTCAYGWCA (SEQ ID NO:7) and WSCARPLCG (SEQ ID NO:8) showed preferential homing to the lymph nodes compared to brain, which was used as a control organ (data not shown).

The immunogenicity of phage displaying lymph node targeting peptides PTCAYGWCA (SEQ ID NO:7) or WSCARPLCG (SEQ ID NO:8) was compared to that of the insertless control phage (fd-tet phage). Mice immunized with lymph node homing phage consistently exhibited a markedly higher titer of anti-phage antibodies than mice immunized with fd-tet phage (FIG. 7). Mice immunized with lymph node-homing phage were pre-injected with the cognate synthetic peptides (PTCAYGWCA, SEQ ID NO:7; WSCARPLCG, SEQ ID NO:8; or vehicle as control). Anti-phage antibody serum titers in mice vaccinated with lymph node homing phage alone were again higher than those seen in mice vaccinated with fd-tet phage. However, mice pre-

treated with cognate synthetic peptides and then vaccinated with lymph node-homing phage had a titer similar to fd-tet-immunized mice (FIG. 8). The results of this example show that this effect is mediated by accumulation of phage in the lymph node due to receptor mediated homing.

Antigens targeted to the endothelium of lymph nodes enhanced the humoral immune response after vaccination. The skilled artisan will realize that this novel method of modulating humoral immune response by attachment of an antigen to a lymph node targeting peptide may be of general utility for enhancing the efficacy of vaccines against a variety of pathogenic agents or diseased cells. For example, attachment of lymph node targeting peptides to tumor associated antigens (TAA) might be used to enhance the systemic humoral response to human tumors. Attachment of lymph node targeting peptides to a wide range of inactivated pathogens, such as bacteria, viruses, retroviruses, HIV, unicellular organisms, or multicellular pathogenic organisms might be of use for enhancng the immune system response to pathogenic challenge. This approach may be further modified by the targeted delivery to lymph nodes of immune system regulatory molecules, such as cytokines or chemokines, either alone or in combination with targeted delivery of specific antigens. Recently, striking cytolytic responses against HIV-1 were observed by displaying viral peptide epitopes within the phage capsid (De Berardinis *et al.*, 2000). Combining this approach with immune system modulation may improve vaccine efficacy and immunotherapy against a host of infectious and malignant diseases.

* * *

All of the COMPOSITIONS, METHODS and APPARATUS disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it are apparent to those of skill in the art that variations may be applied to the COMPOSITIONS, METHODS and APPARATUS and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it are

apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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WHAT IS CLAIMED IS:

1. A method for modulating immune system response comprising:

   a) obtaining a lymph node targeting peptide;

   b) attaching the targeting peptide to an immunogen; and

   c) administering the targeting peptide and immunogen to an individual.

2. The method of claim 1, wherein the immunogen is a peptide, protein, glycoprotein, lipid, carbohydrate, nucleic acid, prion, virus, bacterium, phage, spore, mold, yeast, algae, amoebae, *Ghiardia*, dinoflagellate, unicellular organism, pathogen, cell or infectious agent.

3. The method of claim 2, wherein the targeting peptide is expressed as part of a surface protein of the immunogen.

4. The method of claim 1, wherein the targeting peptide is attached to a Fab fragment that binds to the immunogen.

5. The method of claim 1, wherein the targeting peptide is bispecific.

6. The method of claim 5, wherein the targeting peptide contains a moiety that binds to the immunogen.

7. The method of claim 1, wherein the targeting peptide comprises the amino acid sequences CAY (cysteine-alanine-tyrosine) or SCAR (serine-cysteine-alanine-arginine).

8. The method of claim 7, wherein the targeting peptide comprises SEQ ID NO:7 or SEQ ID NO:8.

9. The method of claim 1, wherein said administering is effective to increase the individual's immune response to the immunogen, compared to the immune response in the absence of the targeting peptide.

10. The method of claim 9, further comprising vaccinating the individual against the immunogen.

11. The method of claim 9, wherein the immunogen is a cancer cell.

12. The method of claim 9, wherein the immunogen is HIV (human immunodeficiency virus).

13. A bispecific compound comprising the amino acid sequences CAY or SCAR, attached to a second moiety.

14. The compound of claim 13, wherein the second moiety is an antibody or a fragment of an antibody.

15. The compound of claim 14, wherein the antibody or fragment of an antibody binds to an immunogen.

16. The compound of claim 13, wherein the second moiety is a peptide that binds to an immunogen.

17. A bispecific compound comprising a targeting peptide attached to a vector binding moiety.

18. The compound of claim 17, wherein the vector is an adenovirus or an adeno-associated virus (AAV).

19. The compound of claim 17, wherein the vector binding moiety comprises an antibody or Fab fragment of an antibody.

20. The compound of claim 19, wherein the antibody is IC5IIE11 or 3B2ID10.

21. The compound of claim 17, wherein the targeting peptide comprises SEQ ID NO:5.

22. The compound of claim 17, wherein the compound comprises SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 or SEQ ID NO:24.

23. The compound of claim 17, wherein the compound comprises the amino acid sequence ELR (glutamate-leucine-arginine).

24. The compound of claim 17, wherein the compound comprises the amino acid sequence GR (glycine-arginine).

25. A method of targeting a vector to an organ or tissue comprising:

a) obtaining a targeting peptide against the organ or tissue;

b) obtaining a binding moiety against the vector;

c) attaching the targeting peptide to the binding moiety to form a complex; and

d) administering the complex and the vector to a subject.

26. The method of claim 25, wherein the vector is an adenovirus or AAV.

27. The method of claim 25, wherein the binding moiety is an antibody or Fab fragment of an antibody.

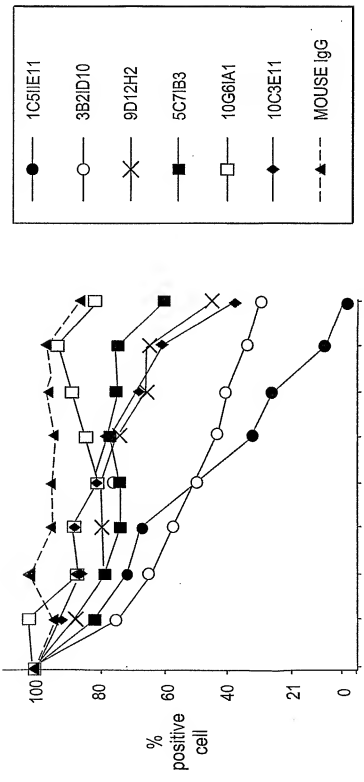
28. The method of claim 27, wherein the antibody is IC5IIE11 or 3B2ID10.

29. The method of claim 25, wherein the targeting peptide and the binding moiety are part of a bispecific compound.

30. The method of claim 29, wherein the bispecific compound is a single peptide.

31. The method of claim 1, wherein the individual is a mammal.

32. The method of claim 31, wherein the individual is a mouse or a human.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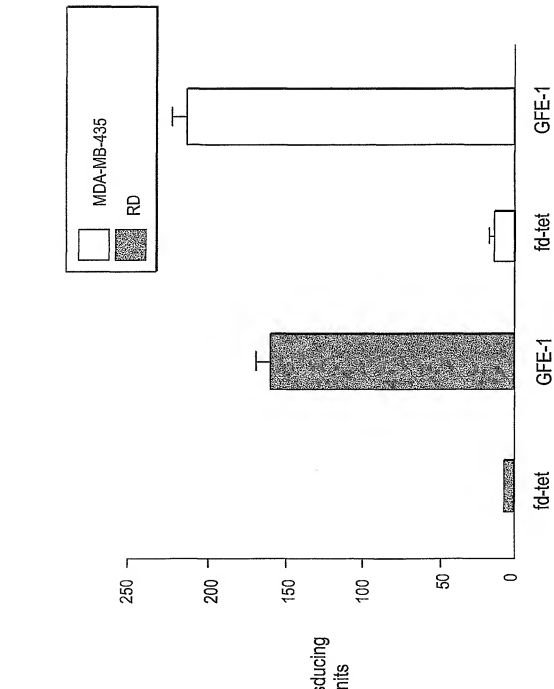
50.0
µg/ml

Fig. 1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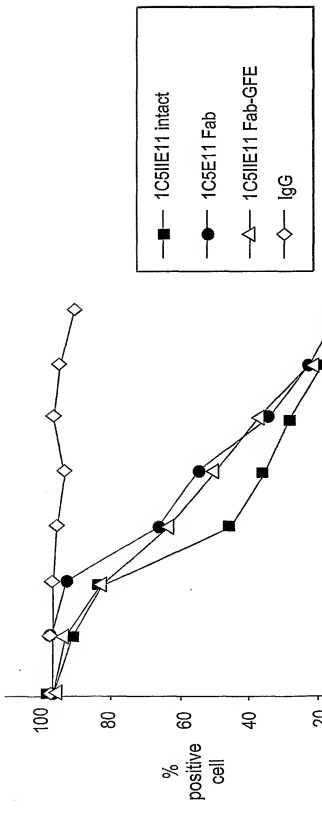
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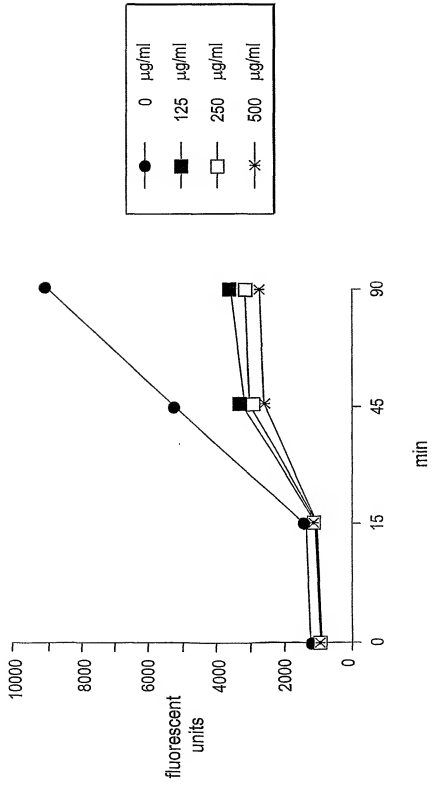
Fig. 3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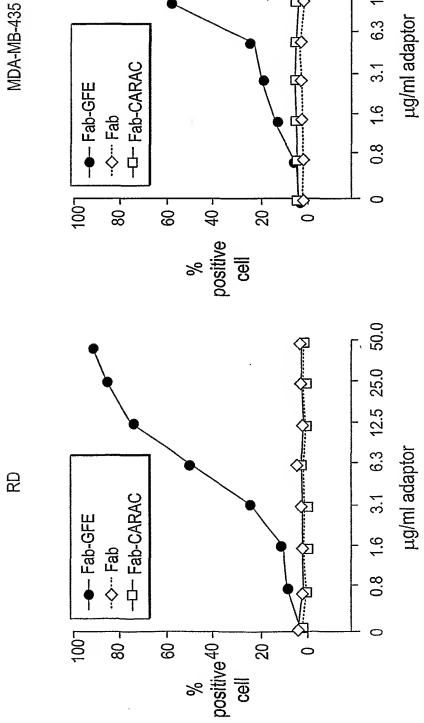
0
0
15
45
90
min

Fig. 4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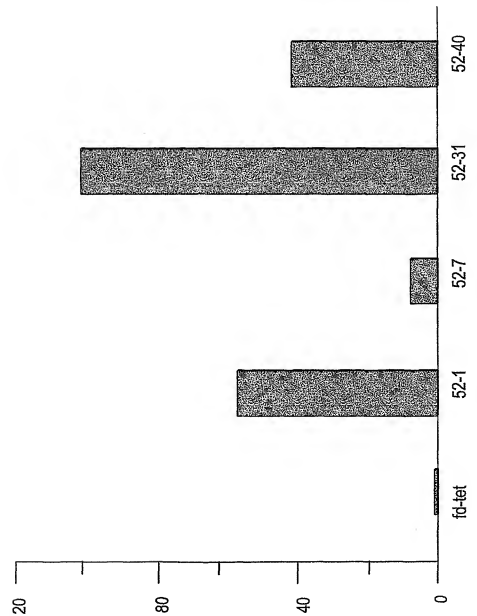
µg/ml adaptor

Fig. 5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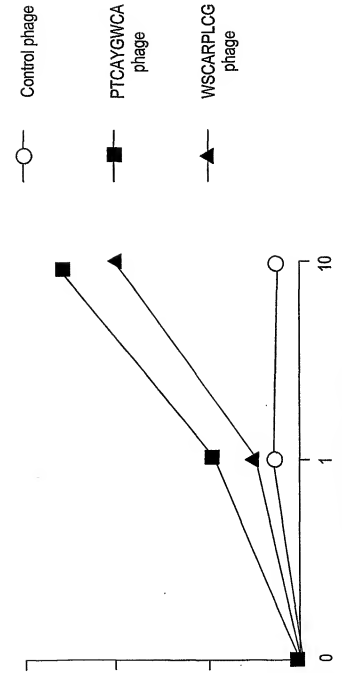
Phage

Fig. 6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0
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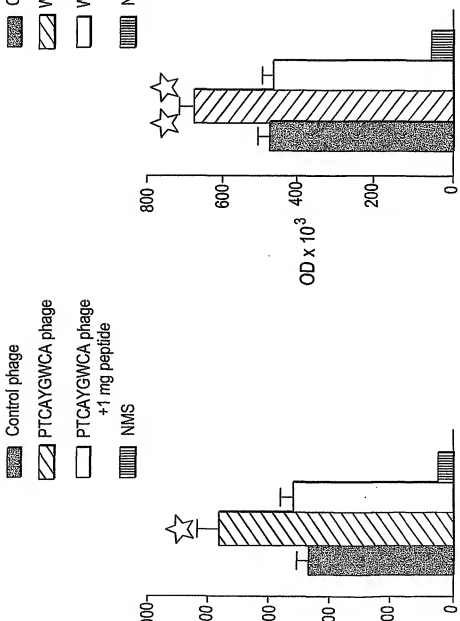
10
TU injected phage x 10^5

Fig. 7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NMS
OD x 10^3
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800
600
400
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Fig. 8

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SEQUENCE LISTING

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